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14. ABSTRACT The goal of this research is to verify the specificity of the inhibition of AR-p52 interaction by small molecule AR/p52 inhibitors (selected from prior high throughput screen) in cell culture and AR/p52 activity assays, and determine the efficacy of the compounds against castration resistant prostate cancer cell / xenograft growth. Data from this research will establish the AR-p52 interaction as a viable new target for preventing progression to castration resistant prostate cancer (PCa) and identify lead compound(s) to be further developed for preclinical toxicity testing and clinical trials for PCa that fall beyond the scope of this proposal. During the first year of the research a lead compound has been established with evidence of specificity for AR-p52 interaction and significant inhibition of both castration resistant and androgen-dependent PCa cell growth, and preliminary evidence of efficacy of the lead compound administered at MTD oral regimen against a castration resistant PCa xenograft model.

15. SUBJECT TERMS androgen receptor, nuclear factor kappa B p52, small molecule inhibitors, human prostate carcinoma cells, castration resistant prostate cancer xenograft efficacy

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INTRODUCTION

About 30% of all prostate cancer (PCa) patients after first line of therapy succumb to recurrent PCa. Although the recurrent PCa regresses after androgen deprivation therapy (ADT), the majority of these patients return to the clinic with the refractory PCa known as castrate resistant PCa (CRPCa). There is no approved drug that can prevent the transition of PCa to CRPCa in these patients. Accumulative evidence suggests that androgen-independent activation of androgen receptor (AR) and development of apoptosis-resistant cells play key roles in the transition of androgen-dependent PCa (ADPCa) to CRPCa (1). AR activation and signaling under very low androgen level or in the absence of androgen may occur by a variety of mechanisms that alter the sensitivity and/or specificity of AR activation. Most of the NF-κB proteins have been shown to be aberrantly activated in PCa cells and tissues (2)]. In the less-explored noncanonical NF-κB2 pathway, protein p52 induces the expression of genes that are involved in hyperplasia, growth and cell proliferation. Overproduction of p52 has been observed in several solid tumors including PCa, and it was recently shown that overexpression of p52 induces castration-resistant growth in human prostate carcinoma LNCaP cell xenografts by inhibiting both cell cycle arrest and apoptotic cell death induced by androgen deprivation (3). Thus, one possible mechanism of aberrant activation of AR in the absence of androgen is its activation by p52. Using a *Gaussia* Luciferase (GL) reconstitution assay (4), we found that AR interacts directly with p52 *in situ* under androgen-deprived conditions. We further developed and used a novel GL reconstitution based high throughput screening assay to identify four drug-like small molecules that specifically inhibit this interaction of AR and p52. We performed a preliminary high throughput screen (HTS) of a 2,800 compound subset of the Life Chemicals Library of drug-like small molecules (5). This screen was performed under an androgen-deprived condition and yielded 296 “hits” which were further screened using a positive control reported in the literature (4) to eliminate the non-specific inhibitors. Only “hits” that inhibited GL-reconstitution in cell lysate from GL1-AR/P52-GL2 cotransfection, but failed to inhibit the control protein-protein interaction were considered “true” hits. These prior studies yielded four compounds that are specific inhibitors of AR and p52 in the absence of androgen (shown in Figure 1 in Appendix). We hypothesize that small molecule inhibitors of the AR-p52 interaction will inhibit aberrant activation of AR by p52 and thereby prevent the transition of ADPCa to CRPCa and growth of CRPCa. The purpose of this research is to verify the specificity of the inhibition of AR-p52 interaction by these compounds in cell culture as well as in AR/p52 activity assays, and investigate the anti-CRPCa activity of the compounds in androgen-independent human LNCaP C4-2 cells and xenografts. Data from this research will help identify lead small molecule(s) to be further developed for preclinical toxicity testing and clinical trials for PCa that fall beyond the scope of this proposal. This report summarizes the progress that has been achieved toward completing the proposed aims.

BODY

The following are the data collected with respect to tasks listed in our statement of work (all figures and tables referred to in this report are included in the Appendix):

<u>Task 1. Investigate the anti-CRPCa activity and specificity of the inhibition of AR-p52 interaction by the four HTS-identified drug-like small molecule AR-p52 inhibitors *in vitro*:</u>

Effect of the AR-p52 inhibitor compounds on the growth of androgen-dependent LNCaP and the castration resistant variant LNCaP C4-2 cells:

We have determined the effects of these four inhibitors on the growth of androgen-dependent LNCaP cells and its castration resistant variant LNCaP-C4-2 cells. Analysis of the effect of these compounds on the castration-resistant (CR) growth of LNCaP C4-2 cells under androgen

deprivation conditions using our published assay (6) showed significant ($P<0.05$) growth inhibition by all four inhibitors in the 1 to 25μM dose range, and compounds AR/p52-01, -02 and -03 had IC_{50}s of 10μM or less (Table 1, see Appendix). Danquah *et al* (7) showed that the IC_{50} of the clinically used bicalutamide (Casodex, Astra-Zenecca; an androgen antagonist that is the current standard ADT) in LNCaP C4-2 cells is 92.9 μM, which is likely a clinically unachievable concentration. Thus, these small molecule inhibitors of AR-p52 interaction show promise for blocking CRPCa growth post ADT. Similar analysis of effect on androgen-dependent (AD) growth of the parental LNCaP cells under growth stimulatory androgen conditions showed AR/p52-01 and -02 had IC_{50}s of 5μM or less (Table 2, see Appendix), with significant growth inhibition in the 1 to 25μM dose range, while compounds ARp52-03 and -04 showed no significant effect on AD LNCaP growth at any dose. Overall, compounds AR/p52-01 and -02 were the most effective for inhibiting these models of PCa cell growth, with IC50s at 4 to 5 μM for both CR LNCaP C4-2 and AD LNCaP growth. Additional studies are ongoing to demonstrate the effect of AR/p52-01 and -02 against growth in another CRPCa cell model, CWR22v-1 PCa cells.

The AR-p52 inhibitors do not bind to the Ligand Binding Domain (LBD) of AR:
It has been shown that NF-κB2/p52 and N-terminal domain (NTD) of AR coimmunoprecipitate (8). Based on this finding, we hypothesized that the inhibitors of this interaction may interfere with binding of NF-κB/p52 to NTD portion of AR and not to its Ligand Binding domain (LBD). We performed a Ligand Binding Competition Assay (using Polarscreen™ AR Competitor Assay kit from Invitrogen). In Figure 2 (see Appendix), we show that these inhibitors do NOT compete with androgen for binding to the LBD of AR and therefore we concluded that the inhibition of interaction between AR and p52 by these inhibitors is not due to their interference with the binding to LBD of AR. Lack of interference with androgen for binding to AR supports the specificity of these compounds for AR-p52 interaction.

The AR-P52 inhibitors reduce the expression of PSA in androgen-dependent LNCaP cells as well as castration resistant LNCaP C4-2 cells:
As PSA protein expression is a marker of AR activity, we performed first a preliminary analysis to determine the ability of these inhibitors to inhibit PSA expression in castration resistant LNCaP-C4-2 cells (data are summarized in Table 3, see Appendix). Compound AR/p52-02 showed the greatest effect in inhibiting PSA secretion with PSA secretion significantly decreased to 22% of control ($P<0.02$). Interestingly, LNCaP C4-2 cells had 15-fold greater PSA secretion compared to parental LNCaP cells under the same androgen-deprived conditions, with values of 6.2 ng PSA/cell and 0.4 ng PSA/cell respectively in this study. These data indicated significant AR transcriptional activity in androgen-independent LNCaP C4-2 cells under androgen-deprived conditions and the ability of the AR-p52 inhibitors to block this activity.

We then picked the compound that had most effect on the reduction of PSA secretion, AR/p52-02, and performed a complete analysis of PSA expression after treatment of both cell lines with this compound using qrtPCR method (Table 4). Data from this study shows that AR/p52-02 inhibitor reduced the expression of PSA 5.6 fold in the absence of R1881 after 72h in LNCaP cells (Table 4.A and B), however this reduction in the presence of androgen in LNCaP cells that are androgen-dependent was only 1.8. In the presence of antiandrogen bicalutamide (Casodex), AR/p52-02 reduced the expression of PSA in LNCaP cells 2.8 fold which is more than reduction of PSA in the presence of androgen. We concluded that as bicalutamide binds to the Ligand Binding Domain (LBD) of AR and inhibits the AR activity, further decrease in PSA

expression in the presence of AR/p52-02 might be due to the binding of AR/p52-02 to the N-terminal of AR and blocking its interaction site with p52 that results in further reduction in AR activity and ultimately lowers the PSA expression. It has been reported before (8) that p52 binds to AR and increases the activity of AR and its binding to the Androgen Response Element III (ARE III) of PSA promoter. This supports that AR/p52-02 can inhibit AR activity to prevent transition of androgen-dependent prostate cancer cells to castration resistance.

The results of PSA expression in castration-resistant C4-2 cells (shown in Table 4.C and D) after treatment with AR/p52-02 were different from those explained above for androgen-dependent LNCaP cells. In the presence or absence of androgen the PSA expression was not significantly affected by AR/p52-02. This indicates that blocking AR-p52 interaction does not affect AR activity in regard to PSA expression in these cells.

Further analyses of the lead compound AR/p52-02, based on cell growth and PSA analyses, were performed to explore its mechanism of action *in vitro*.

Lead compound AR/p52-02 does not affect apoptosis marker Cleaved PARP, but does affect cell cycle marker Cyclin D1:
Western blot analysis for effect of lead inhibitor AR/p52-02 on a marker of apoptosis, Cleaved PARP, and a marker of cell cycle, Cyclin D1 was performed in LNCaP and LNCaP C4-2 cells (Figure 3, see Appendix). There was no effect on cleaved PARP, suggesting the mechanism of action of AR/p52-02 does not involve apoptosis. Western blot analysis of Caspase 3 and Caspase 7 also showed no effect, further supporting that apoptosis is not involved. However, the reduction of Cyclin D1 in C4-2 cells under androgen-deprived condition as well as in both C4-2 and LNCaP in the presence of androgen suggests the mechanism of action of AR/p52-02 involves cell cycle. This will be explored more in depth in future proposals.

Importantly, western blot analysis of LNCaP C4-2 cells treated with or without AR/p52-02 at IC50 value of 5 μM showed no effect of the drug on AR or p52 protein levels, thus indicating that the mechanism of action of AR/p52-02 does not involve a reduction in AR or p52 protein. This further supports the specificity of the compound for inhibiting the interaction of AR and p52.

Based on the results of Task 1, compound AR/p52-02 was prioritized as the lead compound for the proposed *in vivo* studies in Task 2.

<u>Task 2. Investigate the anti-CRPCa activity of up to two compounds selected from Task 1 *in vivo* .</u>

Determination of oral bioavailability and maximum tolerated dose (MTD) for lead compound AR/p52-02:
Compound AR/p52-02 was advanced into animal studies. Solubility of the compound was determined in our standard administration vehicle, 0.9% saline. A preliminary PK / toxicity study established that a single dose of 5 mg/kg in 6%DMSO saline by either intra venous (IV) or oral administration was well-tolerated and yielded plasma levels at 30 minutes post administration of 3.2nM Cmax for IV and .05nM Cmax for oral dosing (Table 5, see Appendix). Thus, the compound was determined to have oral bioavailability, and is therefore an ideal candidate for clinical application. Additional PK and maximum tolerated dose (MTD) studies for a daily oral dosing regimen of this compound were undertaken. Analyses of plasma and tissue samples from PK studies are ongoing. MTD studies established the regimen of 50 mg/kg oral dailyx5 every week, using a 60% DMSO in saline formulation, as MTD for use in efficacy studies.

Efficacy of AR/p52-02 against castration-resistant LNCaP C4-2 xenografts compared to parental androgen-dependent LNCaP xenografts:

To-date, one study of efficacy against the castration resistant LNCaP C4-2 xenograft models of prostate cancer compared to the androgen dependent parental LNCaP model has been completed, and a repeat study to confirm and add statistical power for final analysis is underway. A preliminary analysis of the current data from the study, shown in Table 6 (see Appendix), indicate efficacy of AR/p52-02 against these prostate cancer models. For the parental androgen-dependent LNCaP xenograft model, 24 mice received a subcutaneous implant of LNCaP cells, and two weeks later were randomized to n=12 Vehicle Control (60% DMSO saline) versus n=12 GWARJD10-001 at 50 mg/kg daily oral x 5 treatment every week until sacrifice due to tumor size / decrease in body weight or body condition (Required Sacrifice), or at timed harvest at 10 weeks following initiation of treatment, which was when the majority of remaining Vehicle Control mice required sacrifice due to tumor size/body condition. The data show that treatment with AR/p52-02 led to a 25% decrease in the number of mice requiring sacrifice due to tumor size or decrease in body weight/condition and a nearly 2.5-fold reduction in tumor size at end of study timed harvest. Results were even more striking for the castration resistant (androgen-independent) LNCaP C4-2 xenograft model, which followed the same timeline and treatment regimen (n=7 mice per treatment group): drug treatment yielded a 42% reduction in tumor development, a 43% reduction in number of mice requiring sacrifice, and > 5-fold reduction in average tumor volume at end of study timed harvest. Thus the lead ARp52 inhibitor showed strong activity against this animal model of CRPCa.

In conclusion, we made significant progress in completing the aims of this research within the original one year period of this project. In the 6 month no cost extension that has been granted, we will complete the ongoing repeats of key *in vitro* and *in vivo* experiments for both tasks as well as the animal tissue analyses for task 2, perform final statistical analyses and prepare the data for publication.

KEY RESEARCH ACCOMPLISHMENTS:

- Determined IC50s for the four AR-p52 inhibitors for inhibition of growth of castration-resistant LNCaP C4-2 and androgen-dependent LNCaP human prostate cancer cells; compounds AR/p52-01 and -02 most effective with IC50s of 4 to 5 µM against each model
- Determined that the four AR-p52 inhibitors do not bind the ligand binding domain (LBD) of AR and therefore are not anti-androgens based on an AR-LBD binding competition assay
- Determined ability of the compounds to inhibit AR activity based on analysis of secreted PSA; compound AR/p52-02 most effective at inhibiting PSA secretion
- Demonstrated significant reduction of AR activity by AR/p52-02 based on reduced PSA expression in LNCaP cells
- Detemined lead compound AR/p52-02 does not affect apoptosis based on its lack of effect on Cleaved PARP, but does affect cell cycle based on its reduction of Cyclin D1
- Established that AR/p52-02 has oral bioavailability and is tolerated in mice with MTD of 50 mg/kg administered oral once daily x 5 every week
- Performed first study of efficacy of AR/p52-02 against castration resistant LNCaP C4-2 xenograft growth compared to androgen-dependent LNCaP xenograft growth; repeat study is underway

REPORTABLE OUTCOMES:

- Siefkes E, Church D, Wilding G, and Mehraein-Ghomi F. The effect of four inhibitors of androgen receptor and p52 interaction on the proliferation of prostate cancer cells. Poster Presentation at UW-Madison Biology Undergraduate Mentored Research Symposium, May 2012.
- CDMRP PCRP grant proposal PC131466 "Development of a Novel Method for Detection of Inhibitors of N-Terminal Domain of Androgen Receptor", PI: Farideh Mehraein-Ghomi
- CDMRP PCRP grant proposal PC130353 "Mechanistic Analysis of Inhibitors of NFkappaB/p52 – AR Interaction for Prevention of Castrate-Resistant Prostate Cancer Progression" PI: Farideh Mehraein-Ghomi

CONCLUSION:

In summary, the data accumulated to-date overall demonstrate a specificity of these compounds for inhibiting the AR – p52 interaction and efficacy in blocking both androgen-dependent and androgen-independent (castration resistant) prostate cancer cell growth. The lead compound, AR/p52-02, shows promise in the animal studies performed to-date for oral bioavailability and efficacy against growth of castration resistant prostate cancer xenografts. We anticipate that completion of the experiments for this project and final analysis of results will identify AR/p52-02 as a candidate for further preclinical development toward clinical testing, as well as establish AR-p52 inhibitors as a new class of agents for further research and development as new therapies to prevent progression to CRPCa in PCa patients who underwent androgen ablation therapy.

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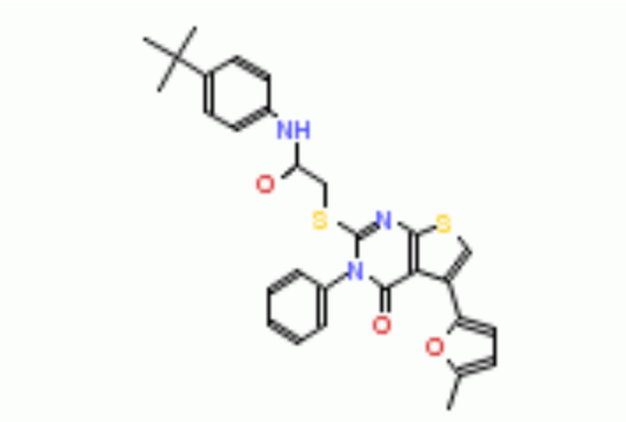
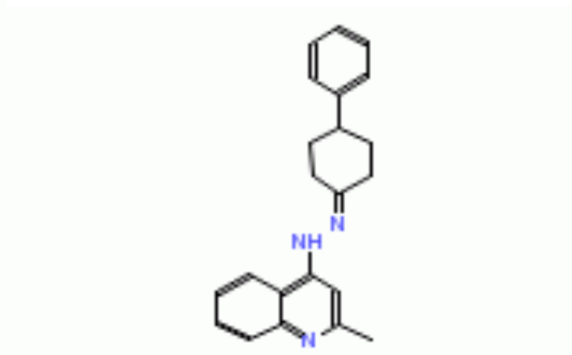
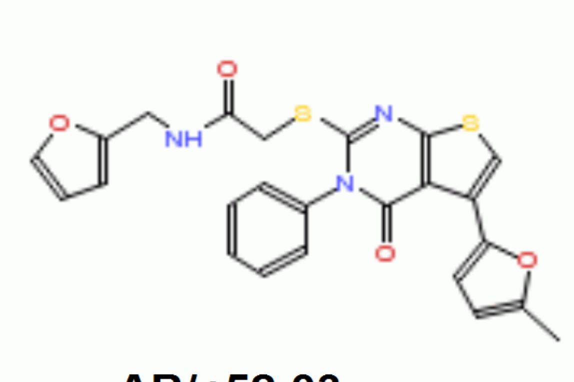
APPENDIX

AR/p52-01



AR/p52-02





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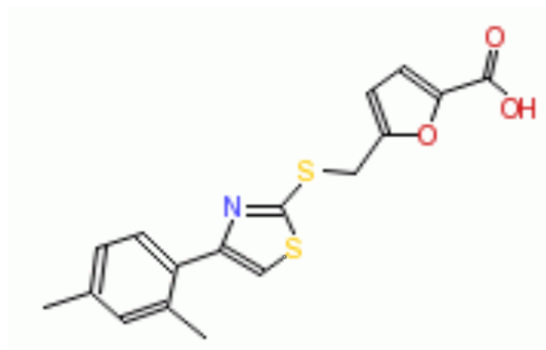


AR/p52-04

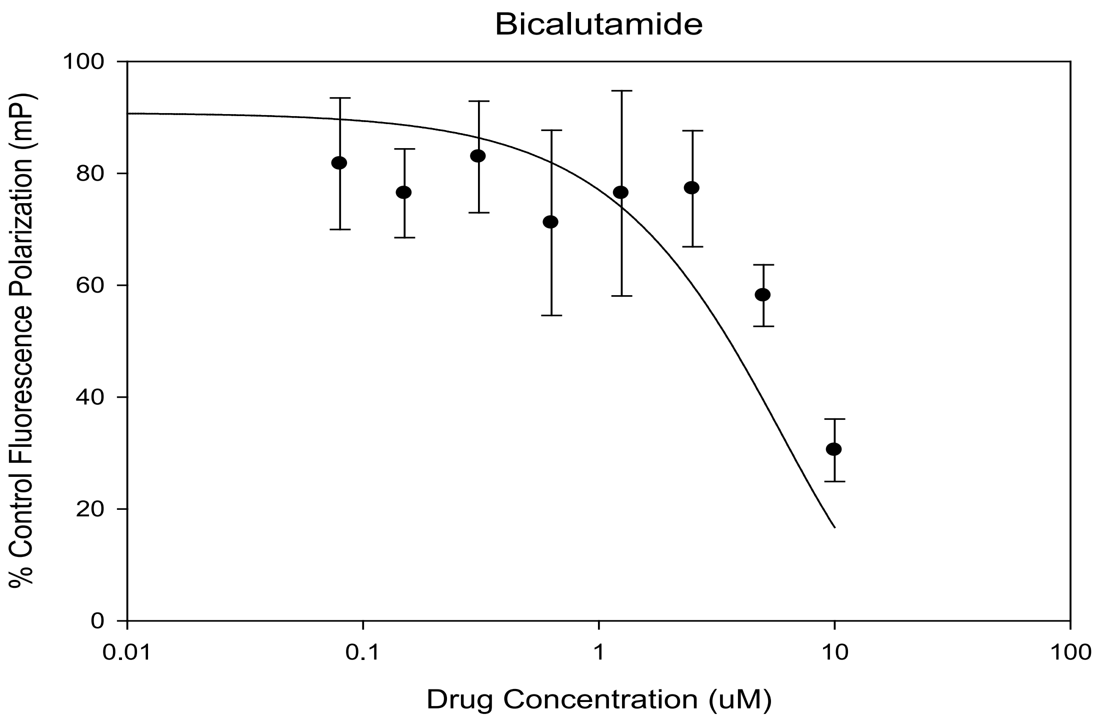
Figure 1. Structure of specific inhibitors of AR and p52 interaction identified by high throughput screen.

Table 1. IC50 values for inhibition of castration-resistant LNCaP C4-2 cell growth by AR-p52 inhibitors under androgen deprived conditions. LNCaP C4-2 cells in androgen-deprived medium were treated with varying doses of compound up to 25 μM or zero dose control for 96 h, then harvested for DNA quantitation and analysis of growth inhibition dose response to determine the dose at which growth was inhibited by 50% compared to control (IC50). N=6 data points per dose, experiments repeated.

Compound	**IC50 (μM)**
ARp52-01001	4
ARp52-02001	5
ARp52-03001	10
ARp52-04001	> 25

Table 2. IC50 values for inhibition of androgen-dependent LNCaP cell growth by AR-p52 inhibitors under androgen growth stimulatory conditions. LNCaP cells in medium containing growth stimulatory level of androgen were treated and analyzed as described above to determine IC50s.

Compound	**IC50 (μM)**
ARp52-01001	4
ARp52-02001	5
ARp52-03001	> 25
ARp52-04001	> 25



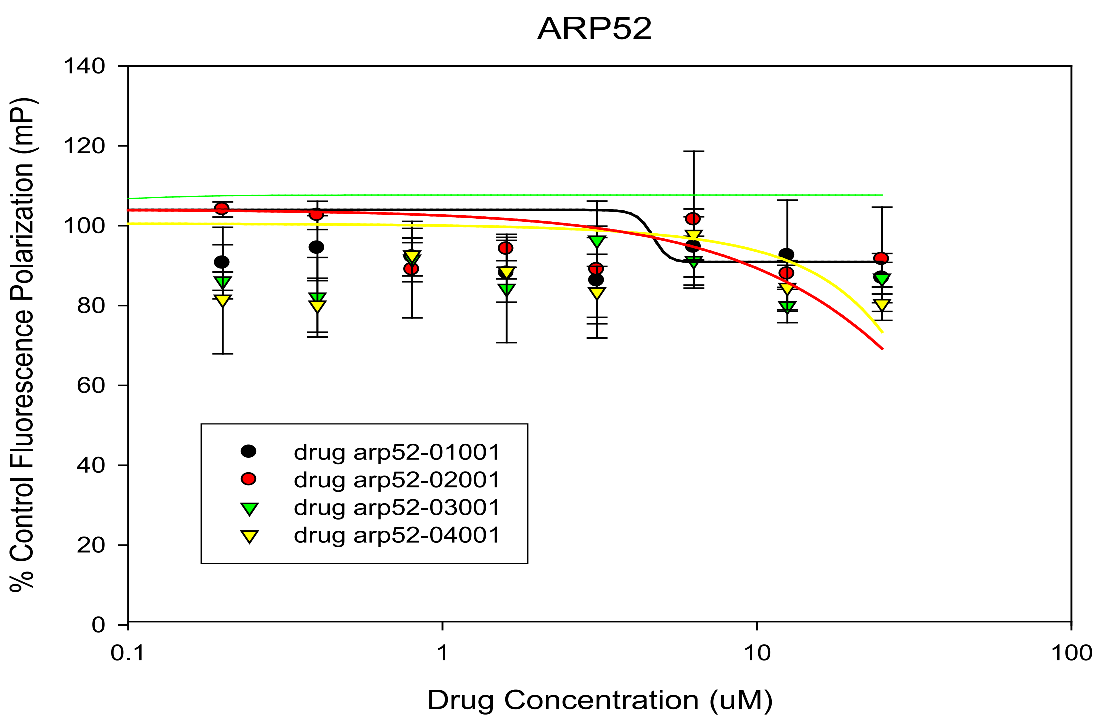


Figure 2. The AR-p52 inhibitors do not bind AR Ligand Binding Domain (LBD). Compounds were assayed using Invitrogen's Polarscreen AR Competitor Assay kit. Clinical antiandrogen bicalutamide showed significant competition with androgen for binding to AR LBD as expected (top graph) while the AR-p52 inhibitors did not (bottom graph).

Table 3. AR-p52 inhibitors at 10µM cause a decrease in LNCaP-C4-2 cells PSA secretion under androgen-deprived conditions. LNCaP C4-2 cells were cultured in androgen-deprived medium and treated with vehicle (control) or 10 µM of compound (N=2 samples per condition). Four days into treatment, media and cells were harvested for determination of PSA secretion per cell. The PSA Enzyme Immunoassay kit from BioCheck, Inc. was used to quantitate PSA in the media, and the ng PSA value was normalized to cell number for each sample. Average ng PSA/cell values for each compound were normalized to the average for Control treated cells (% Control PSA/cell).

Compound	% Control PSA/cell
AR/p52-01	56
AR/p52-02	22
AR/p52-03	60
AR/p52-04	56

Table 4. Fold changes in PSA expression after treatment of LNCaP and LNCaP C4-2 cells with inhibitor AR/p52-02. LNCaP cells **(A,B)** were grown overnight in androgen-deprived medium, then treated with 5μM (IC50) of AR/p52-02 in the presence or absence of 2nM synthetic androgen (±R1881) or 1μM bicalutamide.Total RNAs were extracted after 72h and the cDNA were synthesized and quantitative real time PCR using CFX96 instrument (BioRad) was used to evaluate the level of expression of PSA from each condition. The sequence of PSA primers used are as follows: PSA forward:5'GACCACCTGCTACGCCTCA PSA reverse:5' GGAGGTCCACACTGAAGTTTC. The CT values of PSA was normalized against 18srRNA cDNA. The sequence of primers used for 18srRNA in real time PCR were as follows; 18srRNA forward: 5'CGCCGCTAGAGGTGAAATCT and reverse sequence:5'CGAACCTCCGACTTTCGTT. CT values of four different experiments are given with their averages for androgen/drug treated and 2 CT values with their averages for bicalutamide /drug treated in (**A**). The ratio of each treatment versus base medium are shown in (**B**). Castration resistant LNCaP-C4-2 cells were similarly treated and analyzed (**C,D**).

LNCaP cells 72h

A.

LNCaP cells	CT1	CT2	CT3	CT4	mCT
- R	18.1	18.8	21.58	22.55	20.26
+R	13.2	16.28	17.25	20.93	16.92
- R+5μM 02	18.3	21.56	26.27	24.9	22.76
+ R+5μM 02	19.3	15.54	17.80	18.65	17.82
+bical	19.1	20.57	-	-	19.84
+bical+5μM 02	22.2	20.47	-	-	21.34

B.

LNCaPcells	ratio	Fold change
+R/-R	-3.3	9.8 ↑
+bical+5μM02/+bical	1.5	2.8 ↓
+bical/-R	-0.42	1.3 ↑
+R+5μM02/+R	0.91	1.8 ↓
+bical/+R	2.92	7.5 ↓
-R+5μM 02/-R	2.5	5.6 ↓

C4-2 cells 72h

C.

C4-2 cells	CT1	CT2	CT3	CT4	mCT
-R	20.04	18.9	22.76	22.6	21.08
+R	15.29	14.5	21.23	21.26	18.07
-R+5μM02	19.91	15.4	22.3	25.14	20.69
+R+5μM02	17.2	15.3	20	19.6	18.03

D.

C4-2 cells	ratio	Fold change
+R/-R	-3	8 ↑
+R+5μM02/-R+5μM02	-2.66	6.3 ↑
+R+5μM02/+R	-0.05	1.03 ↑
-R+5uM 02/-R	-0.39	1.3 ↑

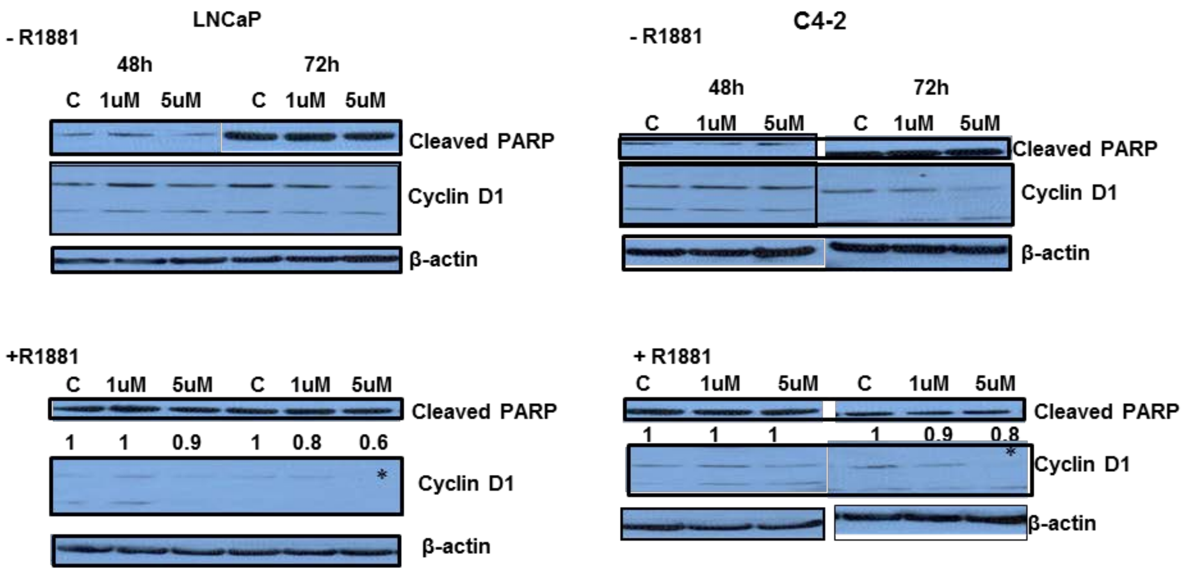


Figure3: Effect of AR/p52-02 on apoptosis marker (Cleaved PARP) and cell cycle marker (Cyclin D1) in androgen-dependent LNCaP cells and castrate-resistant LNCaP C4-2 cells. LNCaP or LNCaP C4-2 cells were treated with (+) or without (-) 2nmol/L androgen R1881 and zero (C), 1 or 5µmol/L of lead compound AR/p52-02 for 72h. Cells were then harvested and protein levels were determined by Western blot analysis. AR/p52-02 had no effect on apoptosis marker Cleaved PARP. AR/p52-02 had a moderate effect on cell cycle marker Cyclin D1 under androgen-deprived conditions (-R1881) in C4-2 cells, and for both LNCaP and C4-2 a significant reduction in the level of Cyclin D1 was observed at 5µmol/L of the compound in the presence of androgen (+R1881) with ratios of 0.6 for LNCaP and 0.8 for C4-2 compared to control (*). This experiment was performed at least three times with similar results.

Table 5. Levels of AR/p52-02 in plasma over time following 5 mg/kg single dose.

Mice were harvested at 30 minutes, 2 hours or 1 week following the single dose administration (N=2 per condition). Plasma was extracted and LC-MS carried out by a standardized gradient of 2% acetic acid water and 2% acetonitrile.

	30 minutes	2 hours	1 week
Oral	.05 nM	.02 nM	0
IV	3.2 nM	1.9 nM	0

Table 6 . Efficacy of AR/p52-02 against prostate cancer xenografts. Athymic nude mice were xenografted with LNCaP or LNCaP C4-2 cells (mice castrated 1 day prior for C4-2), then randomized for initiation of treatment 2 weeks later. Tumor development and growth as well as body weight and condition were monitored over time to determine efficacy. Tumor Development: # mice that developed tumors; Required Sacrifice: # mice requiring sacrifice due to tumor size/ decrease in body weight or condition; End Average Tumor Volume: average size of tumors at timed harvest end of study.

Xenograft Model	Treatment	% Tumor Development	% Required Sacrifice	End Average Tumor Volume (cu.mm)
Androgen Dependent LNCaP	Vehicle Control (n=12)	75	67	434
	50 mg/kg Drug (n=12)	67	42	181
Castration Resistant LNCaP C4-2	Vehicle Control (n=7)	71	57	592
	50 mg/kg Drug (n=7)	29	14	111